Exhibit B

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A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung

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ABSTRACT **Effective gene therapy for lung tissue requires the use of efficient vehicles to deliver the gene of interest into lung cells. When plasmid DNA encoding chloramphenicol acetyltransferase (CAT) was administered intranasally to BALB/c mice without carrier lipids, CAT activity was detected in mouse lung extracts. Plasmid DNA delivered with optimally formulated commercially available transfection reagents expressed up to 10-fold more CAT activity in lung than observed with naked DNA alone. Liposome formulations consisting of (±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE) plus the neutral colipid dioleoylphosphatidylethanolamine (DOPE) enhanced CAT expression by more than 100-fold relative to plasmid DNA alone. A single administration of GAP-DLRIE liposome–CAT DNA complexes to mouse lung elicited peak expression at days 1–4 posttransfection, followed by a gradual return to baseline by day 21 postadministration. Readministration of GAP-DLRIE liposome CAT complexes at day 21 led to another transient peak of reporter gene expression. Histological examination of lungs treated with GAP-DLRIE complexed β-galactosidase DNA revealed that alveolar epithelial cells were the primary locus of expression and that up to 1% of all alveoli contained epithelial cells expressing the transgene.**

The lung is an established site for pharmaceutical intervention (1). Compared with systemic administration, direct intrapulmonary administration is a relatively noninvasive procedure and provides high local concentrations of therapeutic agents. In addition, since the airway penetrates deeply into all areas of the lung, and since the cells lining the airway are relatively accessible, the lung has become an attractive target for gene-based therapies, and in particular for those pulmonary ailments that may be refractory to traditional small molecule intervention.

Cystic fibrosis (CF) is the most widely studied pulmonary disease where gene therapy approaches are being investigated. CF is an inherited genetic disease caused by any of over 400 separate mutations in the gene for the CF transmembrane conductance regulator (CFTR) (2). CFTR is essential for efficient transport of chloride ions and fluid across epithelial cell membranes (3). One therapeutic strategy is based on the introduction of cDNA for normal CFTR into pulmonary epithelia to replace the functionally defective protein. A gene therapy that leads to intracellular expression of the transgene product should ensure accurate processing, protein folding, intracellular transport, and membrane insertion.

Two approaches for introducing transgenes into cells are viral vector-based and plasmid DNA-based systems (4). The adenoviral viral vector strategy typically results in high levels of expression (5). However, when used for *in vivo* applications, the adenoviral vector approach can lead to the generation of host immune responses to viral structural components (6, 7). In some cases, a cellular immune response is generated against adenoviral vector-transfected cells that express heterologous viral proteins (8–10). Such immune responses render the lung refractory to subsequent administrations of adenoviral vector gene therapy.

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Cationic lipids (frequently referred to as cytofectins) can be used to enhance the delivery and expression of plasmid DNA *in vitro* and *in vivo* (11, 12), and a considerable amount of work has been reported using both viral and cationic liposome technologies to transfect lung with a principle application being CF therapy (13–16). Unlike viral vector systems, plasmid based gene therapies using cytofectins appear not to give rise to a specific immune response directed at the delivery system (17–19). In addition, studies of the metabolic fate and toxicity of plasmid DNA have given no indication of untoward systemic effects resulting from either the plasmid DNA or the cytofectin formulation (20, 21). The practical aspects of pharmaceutical drug production also favor plasmid based technologies (4, 11, 12). However, the relatively low levels of *in vivo* plasmid DNA expression obtained with previously described cytofectin reagents somewhat offset the advantages. In this report we confirm previously published results showing that plasmid DNA, without cationic lipids, is expressed at measurable levels in mouse lung (22, 23). Furthermore, we describe a novel cationic lipid, (±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE), which greatly enhances the expression of plasmid DNA delivered to mouse lung.

MATERIALS AND METHODS

Hexane, chloroform, and ethyl ether were from EM Science, and anhydrous ethanol was from Quantum Chemicals (Durham, NC). (±)-3-Dimethylamino-1,2-propanediol was from Janssen Chimica. Dodecyl methane sulfonate was from Nu Check Prep (Elysian, MN). All other synthetic reagents were from Aldrich. Bulk silica G, as well as silica G and GF TLC plates, were from Analtech (nos. 14100, 01521, and 02521, respectively). Amine coated silica TLC plates were from EM Separations (Merck; no. 15647). TLC plates were visualized by spraying with 10% H_2SO_4 in methanol, then charring at elevated temperature (500°C for G and GF plates, 120°C for amine coated plates). Infrared spectra were obtained using a Perkin–Elmer 1310 spectrophotometer. ^{1}H-NMR spectra were from a Bruker (Billerica, MA) AC 300. High resolution mass spectroscopy was performed by the Department of Chemistry at the University of Minnesota using a VC 7070E-HF instrument in FAB mode with PEG as internal standard. For histology, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) substrate solution (no. 5520UC) was from GIBCO/BRL; glutaraldehyde (no. G-5882), $MgCl_2$ (no. M-8266), potassium ferrocyanide (no. P-3289), potassium ferricyanide (no. P-3667), and *N*,*N*-dimethylformamide (no. D-8654) were from Sigma. PCDNA3CAT was purchased from Invitrogen. Dioleoylphos-

Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; GAP-DLRIE, (±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*(dodecyloxy)-1-propanaminium bromide; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; CAT, chloramphenicol acetyltransferase; DOPE, dioleoylphosphatidylethanolamine; β-gal, β-galactosidase; DMRIE, (±)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-*bis*(tetradecyloxy)-1-propanaminium bromide; DOTAP, (±)-*N*,*N*,*N*-trimethyl-2,3-*bis*(z-octadec-9-ene-oyloxy)-1-propanaminium chloride; DOTMA, (±)-*N*,*N*,*N*-trimethyl-2,3-*bis*(z-octadec-9-enyloxy)-1-propanaminium chloride; DOSPA, (±)-*N*,*N*-dimethyl-*N*-[2-(spermin-ecarboxamido)ethyl]-2,3-*bis*(dioleyloxy)-1-propaniminium pentahydrochloride.

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phatidylethanolamine (DOPE) and the cationic lipids EDLPC, EDMPC, and (±)-*N*,*N*,*N*-trimethyl-2,3-*bis*(*z*-octadec-9-eneoyloxy)-1-propanaminium chloride (DOTAP) were obtained from Avanti Polar Lipids. (±)-*N*,*N*-dimethyl-*N*-[2-(sperminecarboxamido)ethyl]-2,3-*bis*(dioleyloxy)-1-propaniminium pentahydrochloride (DOSPA) was obtained from GIBCO/BRL, and DDAB from Eastman Kodak. (±)-*N*,*N*,*N*-trimethyl-2,3-*bis*(*z*-octadec-9-enyloxy)-1-propanaminium chloride. (DOTMA) (43) and DC-cholesterol (37) were synthesized according to published procedures.

GAP-DLRIE Synthesis. A solution of (±)-3-(dimethylamino)-1,2-propanediol (1.77 g, 14.9 mmol) in anhydrous tetrahydrofuran (15 ml) was added dropwise to a slurry of hexane-triturated sodium hydride (60% in oil, 1.52 g, 38 mmol) in anhydrous tetrahydrofuran (100 ml). The reaction was heated to reflux. After 24 hr a solution of dodecylmethane sulfonate (10. 5 g, 38 mmol) in anhydrous tetrahydrofuran (22 ml) was added and the reaction refluxed an additional 72 hr. The reaction was cooled, vacuum filtered through a 1-cm plug of Celite, and evaporated. The residue was partitioned between ethyl ether (250 ml) and 0.2 M sodium hydroxide (50 ml). The organic layer was washed twice with water (50 ml) and then dried over anhydrous magnesium sulfate. Filtration and evaporation afforded crude product. Column chromatography (silica gel, 1:9, ether/hexane to neat ether) afforded clean (±)-(2, 3-di-dodecyloxy)ethyl, dimethylamine (4.40 g, 65%). This amine (2.04 g, 4.48 mmol), anhydrous dimethylformamide (8.5 ml), and *N*-(3-bromopropyl)phthalimide (2.24 g, 8.35 mmol) were combined under an argon atmosphere. The flask was tightly stoppered, then heated to 105°C. After stirring at this temperature for 3 days, the reaction was cooled and the solvent was removed *in vacuo*. Chromatography of the residue (silica gel, 90:10:0.25:0.25, chloroform/methanol/ammonium hydroxide/water) afforded TLC homogenous (±)-*N*,*N*-dimethyl-*N*-(3-phthalimido)propyl-2,3-(*bis*-dodecyloxy)-1-propaniminium bromide (1.99 g, 61%). To a solution of this phthalate (1.99 g, 2.75 mmol) in absolute ethanol (35 ml) anhydrous hydrazine (2.5 ml, 94 mmol) was added. The flask was flushed with argon, tightly stoppered, and stirred at ambient temperature. After 18 hr the thick slurry was diluted with chloroform (70 ml) and then vacuum filtered through a medium glass frit using an additional 70 ml of chloroform as rinse. The combined filtrates were evaporated, and the residue partitioned between chloroform (400 ml) and 0.1 M sodium hydroxide (200 ml). The aqueous phase was washed with chloroform (200, 100, then 50 ml), and the combined organic extracts dried over anhydrous sodium sulfate. Filtration and evaporation afforded a residue which was treated with high vacuum overnight then redissolved in chloroform (10 ml) and filtered through a 0.2 micron PTFE filter. This filtrate was evaporated to afford 1.55 g of waxy crude product. Recrystalization from dry hexane (23 ml) afforded pure GAP-DLRIE (1.23 g, 75%). TLC (amine coated silica gel, 85:15:0.25:0.25, chloroform/methanol/ammonium hydroxide/water, R_f = 0.48). ^{1}H-NMR [300 mHz, $CDCl_3$, tetramethylsilane (TMS)]: δ 4.09 (m, 1H), 3.91–3.81 (m, 3H), 3.72–3.64 (m, 1H), 3.56 (d, J = 3.9 Hz, 2H), 3.47–3.41 (overlapping m and s, 10 h), 2.86 (m, 2H), 1.95v (m, 2H), 1.56 (m, 4H), 1.26 (s, 36H), 0.88 (t, J = 6.6 Hz, 6H). IR (KBr): cm^{-1} 3400 (br), 1575, 1460, 1120, 725. High resolution mass spectrum: $C_{32}H_{69}O_2N_2Br$ (M^+, M − Br) Calc: 513.5359, Found: 513.5336.

Plasmid Constructs. Plasmid VR1332 contains the chloramphenicol acetyltransferase (CAT) reporter gene driven by the human cytomegalovirus immediate early gene I promoter plus intron A with a transcription terminator from the bovine growth hormone gene (24). VR1332 was derived by cloning the CAT gene into *Sal*I/*Bam*HI-digested VR1012 (25). Vector VR1012 is a modified version of VIJ-neo provided by J. Shiver (Merck Sharp & Dohme), which is identical to VIJ (24) except that the ampicillin resistance gene was replaced with the neomycin/kanamycin resistance gene from plasmid puc4K (Pharmacia). Two *LacZ* constructs used in the histochemical studies were derived by cloning a nuclear or a cytoplasmic β-galactosidase (β-gal) gene into VR1012 giving rise to plasmids VR1411 (β-gal-nucl) and VR1412 (β-gal-cyt), respectively. The nuclear β-gal gene was constructed by fusing at the 5′ end of *LacZ* coding region with an oligonucleotide encoding peptide MVKKKRK that resembles the nuclear localization signal in the simian virus 40 large T antigen.

Preparation of Liposomes and DNA Complexes. The cytofectin colipid mixtures were prepared as described (26), by mixing chloroform solutions of the cationic lipids with the chloroform solutions of the colipids DOPE and the cationic lipids EDLPC, EDMPC and (±)-*N*,*N*,*N*-trimethyl-2,3-*bis*(*z*-octadec-9-eneoyloxy)-1-propanaminium chloride (DOTAP) were obtained from Avanti Polar Lipids. (±)-*N*,*N*-dimethyl-*N*-[2-(sperminecarboxamido)ethyl]-2,3-*bis*(dioleyloxy)-1-propaniminium pentahydrochloride (DOSPA) was obtained from GIBCO/BRL, and DDAB from Eastman Kodak. (±)-*N*,*N*,*N*-trimethyl-2,3-*bis*(*z*-octadec-9-enyloxy)-1-propanaminium chloride (DOTMA) (43) and DC-cholesterol (37) were synthesized according to published procedures. DOPE, diphytanoyl-phosphatidylethanolamine, or cholesterol in sterile 2 ml glass vials. The dried lipid films were produced by evaporating the chloroform under a stream of nitrogen, and the vials were placed under vacuum overnight to remove solvent traces. The lipid films, consisting of 1.5 μmol cationic lipid each, were reconstituted with 0.5–1.5 ml sterile pyrogen-free distilled water (GIBCO/BRL) and the vial was vortex mixed for 1 min at room temperature. The cationic lipid/plasmid DNA complexes were prepared by mixing equal volumes of the cationic liposomes (1–3 mM cationic lipid) with the DNA (8–16 mM in water; based on 330 molecular weight per nucleotide monomer).

Transfection of CFT1 Cells *in Vitro*. CFT1 is an immortalized airway epithelial cell line obtained from a CF patient (27). Cells were seeded in 96-well tissue culture plates at 7.5 × 10^3 cells per well and allowed to grow for 5–7 days until confluent. Transfection of CFT1 was performed as described (26) with the following modifications. Appropriate amounts of GAP-DLRIE/DOPE and the plasmid vector pCMVβ (Clontech) in OptiMEM were complexed and 100 μl added to each well. After 6 hr at 37°C, the cells were supplemented with 50 μl of 30% fetal bovine serum (FBS) in OptiMEM and 24 hr later with an additional 100 μl of 10% FBS in OptiMEM. Following a further 24 hr at 37°C, the cells were assayed for expression of β-gal as described (26).

Detection of CAT Expression *in Vivo* Following Intranasal Delivery into BALB/c Mice. Female BALB/c mice (4–6 weeks old) were anesthetized with metaphane and 100 μl of GAP-DLRIE/DOPE/VR1332 was administered. The complexes were delivered by either intratracheal or intranasal catheter. For intratracheal administration, a 22G steel olive tipped catheter containing cationic lipid–plasmid DNA complexes (100 μl) was passed down the upper palate into the trachea. For intranasal administration, a 1-cc syringe with an attached angiocath catheter filled with cationic lipid–DNA complexes (100 μl) was used to administer droplets of the complexes into the nasal passage. The mice were euthanized 2–35 day postinstillation, and the lungs were harvested, frozen, pulverized, and extracted as described (25) and assayed for CAT enzymatic activity. Lungs were homogenized in 240 mM Tris·HCl (pH 7.8) containing 5 mM EDTA, and the cells lysed by three freeze–thaw cycles. The cleared supernatant was heated at 70°C for 20 min to inactivate mammalian deacetylases and then incubated with reaction buffer (150 μl homogenization buffer, 10 μl of 10 mM acetyl CoA and 14 μl ^{14}C-labeled chloramphenicol) for 15 to 60 min at 37°C. CAT activity was then assayed using the method of Gorman *et al.* (28). The variation in these studies was about 3-fold between the highest and lowest expressing lungs in each group.

X-Gal Histochemical Staining and Cell Counting. Mice were sacrificed at 3 days following lipid–DNA administration (n = 13 animals) by sodium pentobarbital overdose and the tracheas were exposed. A tubing connected to a two-way stopcock was fed into a trachea and the air in the lungs was drawn out and OCT embedding media (Tissue-Tek) was injected into the lungs. The lungs were then dissected, embedded in OCT compound, and frozen in liquid nitrogen. Each lung was partitioned into four quarters of top and bottom and left and right lung halves. Each quarter was then cut into 10-μm sections, fixed in 1% glutaraldehyde, washed three times with PBS, and incubated in X-Gal reaction mixture containing 400 μg/ml X-Gal (GIBCO/BRL), 2 mM $MgCl_2$, and 5 mM potassium ferricyanide in sodium phosphate buffer (pH 7.3) at 37°C for 18 hr. The X-Gal was dissolved in dimethyl formamide at 20 mg/ml and then diluted into the reaction mixture.

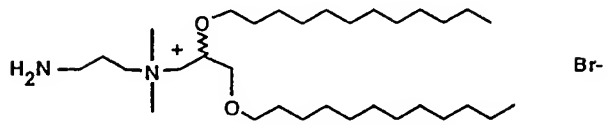

FIG. 1. GAP-DLRIE structure.

The number of X-Gal positive and negative alveoli in randomly selected sections was counted by using light microscopy and a calibrated ocular grid. In cross section, each alveolus was bordered by 2–5 alveolar epithelial cells. An alveolus was scored as (+) if it contained 1 or more X-Gal (+) alveolar epithelial cells.

RESULTS

GAP-DLRIE Chemistry. The structure of GAP-DLRIE is shown in Fig. 1. The basic skeleton of GAP-DLRIE is typical of the 2,3-dioxy-propaniminium class of cationic lipids which also includes (±)-*N*-(2-hydroxyethyl)-*N,N*-dimethyl-2,3-*bis*(tetradecyloxy)-1-propanaminium bromide (DMRIE), DOTAP, DOTMA, and DOSPA. This class of cationic lipids has two hydrophobic chains appended to a quaternary ammonium moiety via a polar dioxy-propyl group in a manner affording a central glycerol-like structure. GAP-DLRIE is readily prepared from commercial reagents in about 30% overall yield (see *Materials and Methods*).

Enhanced Transgene Expression in Mouse Lung. The amount of CAT expressed was determined after intralung administration of plasmid either alone or as plasmid/cytofectin complexes. A total of 130 μg plasmid either alone or complexed with a cytofectin was administered intranasally in 100 μl water to anesthetized BALB/c mice. After 2 days, the lungs were harvested and assayed for CAT. A comparison of two CAT plasmids (Fig. 2) showed that naked VR1332 was 18-fold more active than a commercial pCDNA3CAT construct, and 9 of the 12 cytofectin formulations tested enhanced the level of expression obtained with VR1332 alone. An optimized GAP-DLRIE/DOPE formulation yielded nearly 13 ng CAT per 100 mg lung tissue, representing a 140-fold increase in expression over plasmid DNA alone. It is noteworthy that an optimized formulation of DOSPA/DOPE (1.5:1, cytofectin/plasmid) gave about 11 times the naked DNA signal, while a DOSPA formulation stoichiometrically equivalent to that illustrated for GAP-DLRIE (1:4, cytofectin/plasmid) substantially decreased the expression from the naked plasmid. All other formulations yielded activity less than 4 times that of naked DNA.

Colipid Optimization. Cationic lipids generally produce better transgene expression when mixed with a colipid prior to complexing with plasmid DNA (11). DOPE is an effective colipid for facilitating Rosenthal-type cationic lipid transfection of cells *in vitro*, and the molar ratio of DOPE to cytofectin has been an important variable (26). Here, the transfection efficiency of GAP-DLRIE, formulated alone or at three different molar ratios of DOPE, was examined in a standard *in vitro* β-gal microassay with the CFT1 cell line, and in the *in vivo* murine lung assay using both intranasal and cannula intubation. The results of these assays are shown in Fig. 3. Fig. 3*A* indicates that a 1:3 molar ratio of GAP-DLRIE/DOPE was the most effective in CFT1 cells *in vitro*, while Fig. 3*B* shows that a 1:1 molar ratio was the most effective *in vivo*. The *in vivo* DOPE optimization was the same whether the formulation was administered intranasally or by intratracheal instillation. However, intranasal administration resulted in a higher level of expression than intratracheal instillation. Further *in vivo* studies using intranasal instillation were conducted to determine whether other neutral colipids would substitute for DOPE. Diphytanoyl-phosphatidylethanolamine substituted for DOPE at a similar optimal content of 1:1 molar ratio, however its potency was about 3-fold less than DOPE, and DOPC and cholesterol also produced a very slight enhancement in transgene expression, but their potencies were about 15-fold less than DOPE (data not shown).

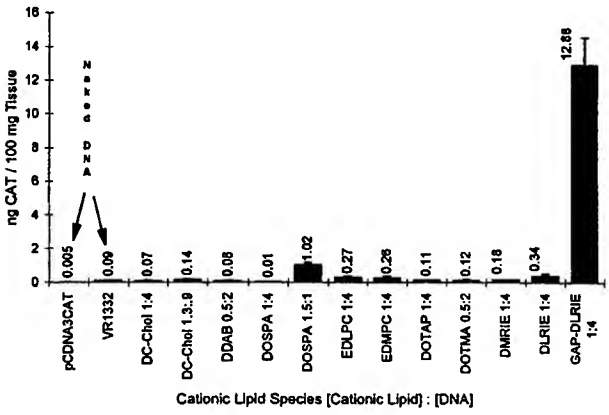

FIG. 2. Comparison of naked DNAs alone or complexed to selected cationic lipid formulations. Groups of five BALB/c mice were dosed intranasally. In all cases the CAT plasmid DNA concentration was 4 mM (1.3 mg/ml). The DNA concentration is based on a molecular weight of 330 for an average nucleotide monomer. For the DC-cholesterol formulation, the cationic lipid concentration was 6 mM. For the GAP-DLRIE formulation the cationic lipid concentration was 1 mM. All formulations were prepared in water. All of the formulations contained DOPE at the same concentration as the cationic lipid. The animals were sacrificed on day 3 postadministration and the lung tissues were homogenized and assayed for CAT activity.

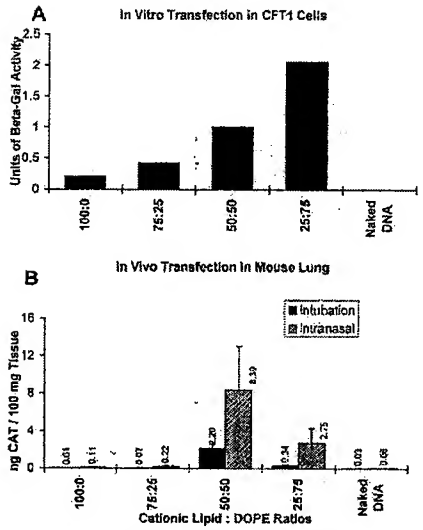

FIG. 3. DOPE optimization. (*A*) *In vitro* transfections were conducted in 96-well microtiter plates containing CFT1, a human CF airway epithelial cell line. The transfections were conducted according to published methods (26). The sum of each of the values in the transfection assay are shown. Four different GAP-DLRIE formulations prepared at four GAP-DLRIE/DOPE molar ratios (100:0, 75:25, 50:50, and 25:75) are shown. (*B*) *In vivo* transfections were conducted using GAP-DLRIE formulated with DOPE at four different ratios. Groups of five animals each were dosed with 100 μl of the formulations consisting of 1 mM cationic lipid and 4 mM DNA. Two different routes of administration, intranasal and intubation, are shown.

DNA and Cationic Lipid Concentration Optimization. To determine the dose concentration of DNA and cationic lipid required for optimal *in vivo* activity, nine formulations were prepared at different DNA and cationic lipid concentrations. Each

Table 1. GAP-DLRIE and DNA dose concentration optimization

	GAP-DLRIE		
DNA, mM	0.5 mM	1.0 mM	1.5 mM
4	1.1 ± 0.5	4.9 ± 0.4	4.4 ± 1.3
6	4.3 ± 0.2	4.9 ± 0.8	4.1 ± 1.8
8	5.2 ± 2.3	2.4 ± 0.4	4.0 ± 1.7

Formulations were prepared at the concentrations indicated, and 100 μl of each formulation was delivered intranasally to anaesthetized mice. The lungs were assayed for CAT activity 3 days postadministration and the values were expressed in μg CAT/100 μg tissue. The difference between the 0.5 and 1.0 mM GAP-DLRIE/DOPE (50:50) formulations at 4 mM DNA was statistically significant ($P = 0.03$) by the Mann–Whitney test.

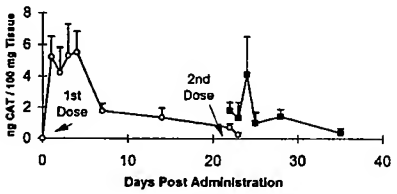

FIG. 4. Time course and repeat dose. BALB/c mice were instilled intranasally on day 0 with 100 μl of GAP-DLRIE/DOPE/VR1332 (0.5:0.5:8). Groups of four mice were sacrificed and their lungs assayed for CAT activity at the indicated times postadministration. A group of mice that was treated on day 0 was given a repeat dose on day 21, and subsets of these animals were sacrificed on the indicated days after the first administration. The difference on days 22 and 23 between the animals given a single dose and those receiving two doses was statistically significantly different ($P = 0.03$) by the Mann–Whitney test.

formulation was screened for *in vivo* reporter gene expression. All animals received an intranasal volume of 100 μl, so that the animals receiving different concentrations of DNA and of cationic lipid received a correspondingly different total dose of each agent. The results are shown in Table 1. For the three formulations prepared at a low concentration (0.5 mM) of GAP-DLRIE, there was a DNA dose-dependent increase in expression when the DNA concentration was increased from 4 mM to 6 mM. Increasing the GAP-DLRIE concentration from 0.5 mM to 1.0 mM improved the efficacy of the 4 mM DNA formulation significantly. All other formulation conditions elicited comparable levels of transgene expression, indicating that expression was relatively insensitive to the formulation conditions over the tested range of cationic lipid and DNA concentrations.

Time Course of Expression and Repeat Administration. A large group of mice received a single intranasal dose of a GAP-DLRIE/DOPE/VR1332 plasmid (0.5:0.5:8 mM). Groups of four animals were sacrificed at 1, 2, 3, 4, 7, 14, 22, and 23 days, and the CAT activity was determined in lung extracts (Fig. 4). Abundant CAT expression could be seen by day 1, and this level was maintained for at least 3 additional days. By day 7, expression had declined to about 30% of day 1 levels, and a further gradual decline was seen until day 23, when most of the remaining CAT had disappeared. Groups of animals that received a single dose on day 0 were given a second dose on day 21, and CAT was measured at days 22, 23, 24, 25, 28, and 35 (corresponding to the first six time points examined after the first administration). There was a statistically significant increase in expression on days 1, 2, and 3 after the second dose, compared with the animals receiving only a single dose. Expression levels declined to near baseline levels by 14 days after the second dose.

***In Situ* Localization of Reporter Gene Expression.** A histological examination of X-Gal-stained cryostat sections was conducted of murine lungs transfected with two different plasmids encoding the β-gal protein, with (β-gal-nucl) or without (β-gal-cyt) a nuclear localization signal. Representative photomicrographs of X-Gal stained lung sections are shown in Fig. 5. Fig. 5 *A–C* are from animals that were administered the plasmid encoding cytoplasmic β-gal, and Fig. 5 *D–F* are from animals dosed with the plasmid encoding nuclear localized reporter gene. In many cases the cytoplasmic expression appeared to spread over an entire alveolus or several alveoli, whereas the nuclear localized β-gal was confined to the alveolar cell nuclei. The predominant morphology of X-Gal(+) lung cells is consistent with the morphology of perialveolar epithelial cells (29) and not with the morphology of alveolar macrophagic cells (30). The number of alveoli containing X-Gal positive cells was determined for each section and the data are shown in Table 2. A total of 1282 sections were analyzed from 10 mice receiving GAP-DLRIE/DOPE liposome lacZ DNA (VR1411, VR1412) complexes. Of these, 960 sections (≈75%) exhibited cellular β-gal expression. β-Gal expression was observed in the lungs of all of the animals receiving intranasal GAP-DLRIE/DOPE/pLacZ complexes. Within each lung (numbered 1–10), the percentage of alveoli containing at least one X-Gal(+) epithelial cell varied from 0.05% to 1.15% of the total number of alveoli per section. No X-Gal staining was evident in control lungs receiving GAP-DLRIE/DOPE liposome CAT DNA complexes (lung numbers 11, 12, and 13). No X-Gal staining was observed with naked VR1332 CAT plasmid alone (data not shown).

DISCUSSION

Reporter gene expression has been observed *in vivo* after naked DNA administration to lung (22, 23), heart (31), liver (32), skin (33), vascular endothelium (34), tumor (35), and numerous tissues after systemic administration (36). Thus, to establish that a given formulation improves *in vivo* plasmid delivery and expression, a naked plasmid DNA control must be used. In the present work, a new cationic lipid molecule, GAP-DLRIE, is described that can enhance functional *in vivo* gene delivery in mouse lung by more than 2 orders of magnitude over that achieved by delivery of naked DNA. GAP-DLRIE emerged as one of the most potent of more than 100 compounds tested for their ability to enhance the expression of the CAT reporter gene in mouse lung following intranasal administration. The transfection activity in mouse lung of GAP-DLRIE was compared with two cytofectins now in clinical use (DMRIE and DC-cholesterol), and a variety of commercially available reagents (DDAB, DOSPA, EDLPC, EDMPC, DOTAP, DOTMA), and with DLRIE another compound from our laboratory that was previously described (12). All of the compounds examined exhibited substantial *in vitro* activity but only one of these (DOSPA) showed activity in lung that approached even 10% of that achieved with GAP-DLRIE. GAP-DLRIE was at least 50 times more active than the other commercially available cationic lipids tested. Thus, GAP-DLRIE provided 140-fold more activity than plasmid alone and was clearly superior to all other cationic lipids tested. There was no obvious correlation between the *in vitro* activity of the compounds and their *in vivo* activity in mouse lung.

The persistence of CAT expression following transfection with GAP-DLRIE/DOPE/pDNA formulations *in vivo* was also assessed. High levels of expression were observed at days 1–3, which declined rapidly to about 30% of the maximum level by day 14. Presently, it is unclear whether this decline in expression was due to loss of plasmid, down-regulation of the promoter driving CAT expression, cytolytic immune response to CAT protein or all three. Nevertheless, these results indicate that expression lasting 2–3 weeks can be achieved using cationic lipid-mediated gene transfer. This observation is consistent with that reported recently by Caplen *et al.* (37) using DC-cholesterol, where a transient bioelectric correction in CF patients was shown after delivery of CFTR plasmid DNA. However, our results and those of Caplan *et al.* (37) are inconsistent with results by McLachlan *et al.* (38) who reported peak CAT reporter gene activity at day 17 following transfection with DOTAP into mice.

There are several reports of reduced efficacy of expression following repeated doses of adenovirus vectors (6, 7, 8, 39). This has been attributed to a host immune response against adenoviral antigens. In contrast, repeat transfection was obtained using GAP-DLRIE/DOPE/CAT complexes, suggesting that an immunological response may not limit the repetitive utilization of this type of synthetic vector. Several studies have been conducted with cationic lipid–plasmid DNA formulations to look for immune responses against cationic lipid or plasmid DNA, and none have been found (18, 20). Heterologous antigens and reporter genes, such as CAT, expressed from plasmids injected intramuscularly have been shown to generate immune responses against the expressed antigens, and these responses limit the expression level obtained from subsequent administrations (ref. 25 and J.H., unpublished data). GAP-DLRIE/DOPE-mediated delivery of a plasmid encoding influenza hemaglutinin into mouse lung leads to an immune response directed against hemaglutinin (J. Donnelly, personal communication). By inference, the GAP-DLRIE-mediated CAT expression reported here would be expected to generate an immune response to the reporter gene, which could lead to a decrease in expression from subsequent administrations. This may account for the lower level of expression observed in the animals that received a second dose of the CAT plasmid three weeks after the first administration. The majority of CF patients receiving CFTR gene therapy express CFTR as a homologous protein and may therefore not develop an immune response to the product of the newly introduced gene, unless the correct form of

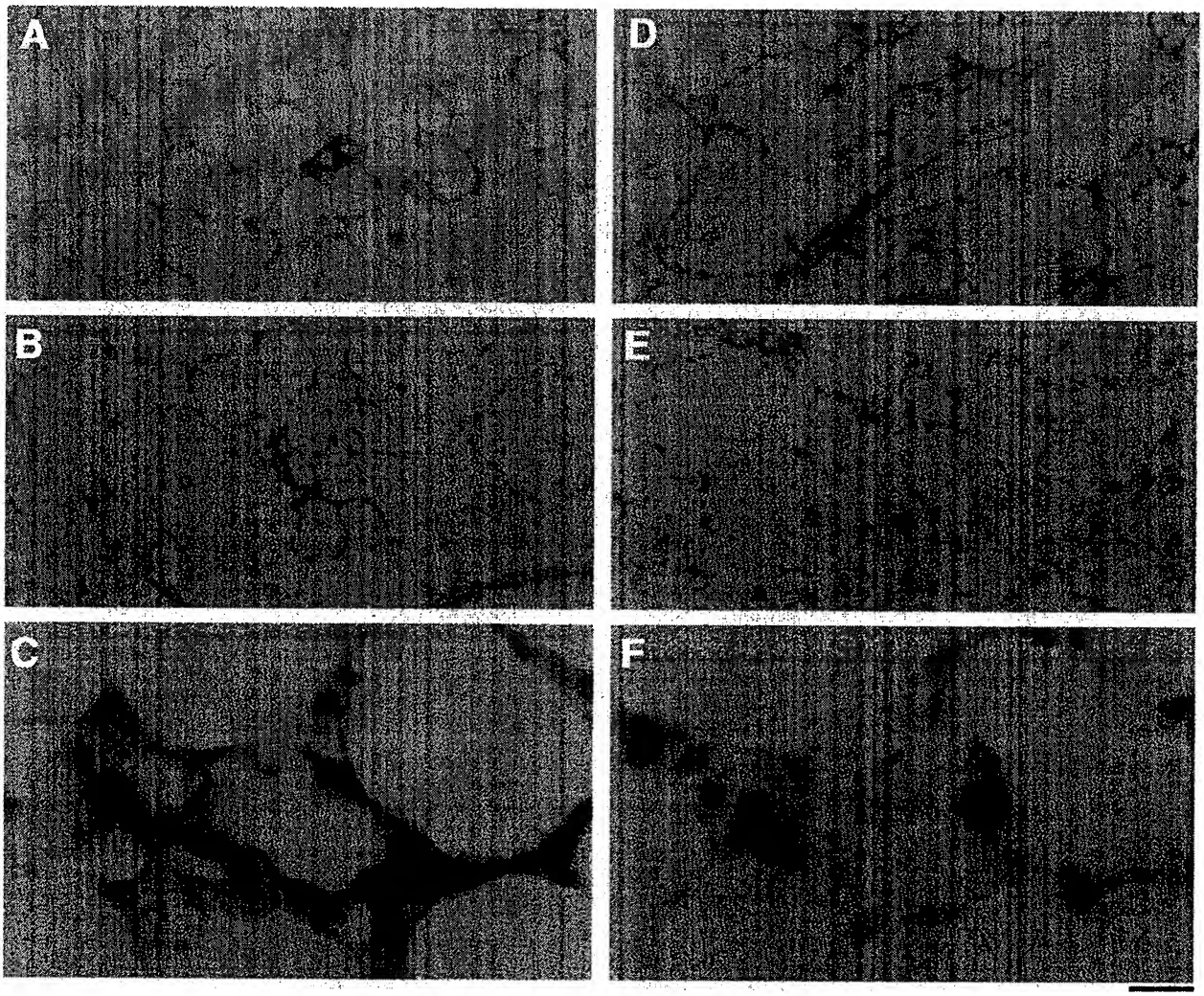

FIG. 5. β-Gal expression in mouse lung. Three days after intranasal administration of GAP-DLRIE complexed with *LacZ* DNA encoding cytoplasmically or nuclear targeted β-gal or CAT (control), mouse lungs were cut into quarters and the quarters separately embedded for cryostat sectioning. Random sections from each quadrant of 13 mice in each group were processed for X-Gal staining, and representative sections shown. The areas shown are from the alveolar space where the majority of X-Gal positive cells were observed. *A–C* are from animals dosed with the cytoplasmically localized *LacZ*, and *D–F* are from the nuclear localized *LacZ*-dosed animals. (Scale bars: *A*, *B*, *D*, and *E* = 0.04 mm; *C* and *F* = 0.01 mm.)

CFTR were to contain immunogenic epitopes previously unseen by the CF patients immune system.

β-Gal plasmids were used to identify the expressing cells in lung after administration of the GAP-DLRIE formulation. The ma-

Table 2. Quantification of *LacZ*-transfected cells in mouse lung

Lung no.	DNA infused	No. of sections examined	No. of X-Gal (+) sections (%)	Average no. of X-Gal(+) alveoli/section	Average total no. of alveoli/section	Average % X-Gal(+) alveoli/section
1	β-gal-cyt	87	38 (44)	4	3424	0.12
2	β-gal-cyt	158	111 (70)	40	7072	0.57
3	β-gal-cyt	120	55 (46)	23	3384	0.68
4	β-gal-cyt	387	332 (86)	25	15111	0.17
5	β-gal-cyt	165	144 (87)	40	7040	0.57
6	β-gal-nucl	24	6 (25)	2	2144	0.1
7	β-gal-nucl	109	104 (95)	76	6593	1.15
8	β-gal-nucl	36	33 (92)	5	2603	0.19
9	β-gal-nucl	42	33 (78)	4	8291	0.05
10	β-gal-nucl	154	104 (67)	36	8246	0.44
11	CAT	30	0 (0)	0		
12	CAT	44	0 (0)	0		
13	CAT	6	0 (0)	0		

Two days after intranasal administration of GAP-DLRIE complexed with *LacZ* DNA encoding cytoplasmically or nuclear targeted β-gal or CAT, mouse lungs were cut into quarters and the quarters separately embedded for cryostat sectioning. Random sections from each quadrant of all 13 mice were processed for X-Gal staining. Data are expressed as the total number of random sections examined per lung, number of sections that contained any X-Gal(+) cells, the number of alveoli containing at least 1 X-Gal(+) cell, the average total number of alveoli present per section, and the average percent of X-Gal(+) alveoli per section.

jority of staining appeared to be localized in the alveolar epithelial cells, and few X-Gal-positive regions were observed in the larger bronchiolar airway cells. It is unclear whether the degree of specificity in transfection of alveolar cells in the present experiments reflects delivery aspects, or whether it represents preferential cellular transfection. It is also unclear just how much and how widely a therapeutic transgene such as the CFTR gene needs to be introduced to offer a therapeutic benefit, although some estimates have been made based on conductance data from *in vitro* systems (39). In addition, the target cells for a CF therapy have yet to be defined, although epithelial cells lining the lower airways and in submucosal glands have been suggested as regions where correction of the chloride imbalance will lead to a CF treatment (40).

GAP-DLRIE is a member of the 2,3-dioxy-propaniminium cytofectin class which also includes DMRIE, DOTAP, DOTMA and DOSPA, and it is, therefore, a member of the most widely used skeletal type of cytofectins. GAP-DLRIE possesses fully saturated and substantially shorter alkyl chains than DOTAP, DOTMA or DOSPA, which should result in a more water-soluble, less hydrophobic molecule, with a higher critical micelle concentration and a higher spontaneous rate of monomer lipid transfer through the aqueous phase. These physical properties will also be influenced by the presence of a primary amine appended to the quaternary ammonium group in the polar head region of GAP-DLRIE. Protonating the primary amine would provide a formal charge of +2 to GAP-DLRIE and increase the hydrophilic character of the compound relative to singly charged cytofectin analogs. Using the second pKa of propylene diamine (9.3) as a model, an equilibrium between the protonated and deprotonated forms of GAP-DLRIE in liposomes might be anticipated at the physiological pH of 7.4. The degree of protonation of GAP-DLRIE liposomes will be affected by surface pH which is expected to be 3–4 pH units higher than the bulk pH (41). Thus, when cytofectin–DNA complexes comprising GAP-DLRIE enter into the low pH endosomal compartment, their protonation state and physical organization may change leading to release of DNA from the endosome. Similarly, the amine may act as a base at an endosomal pH of 5, thereby augmenting plasmid delivery to the cytoplasm in a manner postulated for lysosomotropic agents (26). Certain consequences of introducing a single primary amine into a cytofectin have recently been revealed (42). Thus, the shorter alkyl chains of GAP-DLRIE, and its complex polar headgroup consisting of quaternary and primary amino groups, may together impart physical properties to the cationic lipid–DNA complexes which result in optimal, (*i*) DNA–cationic lipid interaction and lipid–DNA packing, (*ii*) cell surface interaction, (*iii*) endosomal escape (or endosomal avoidance), and (*iv*) intracellular dissociation of the cationic lipid–DNA complexes.

Numerous cationic lipids that have been shown to be highly effective for *in vitro* (26, 43–45) transfection are poorly active *in vivo*. In contrast, the level of expression obtainable with the newly designed cationic lipid molecule, GAP-DLRIE, can reach 450 times the level obtainable with naked plasmid DNA in some animals. The results reported here also indicate that the ability of specific cationic lipids to enhance *in vivo* expression of plasmid DNA must be determined with reference to the target cell in the particular tissue to which the DNA is being administered. Similarly, the amount of DOPE required for optimal *in vitro* and *in vivo* activity differ and, therefore, the efficacy of cationic lipid formulations for *in vivo* gene delivery to lung cannot be straightforwardly predicted from *in vitro* transfection assays, and must also be determined by direct *in vivo* assay.

Several clinical studies using plasmid DNA based vectors for the treatment of CF patients are underway or completed (10, 18, 37). The results reported here suggest that an optimal cationic delivery vehicle will elicit a dramatic enhancement in DNA delivery and expression. Future plans include testing lipids of this type in a clinical setting to ascertain whether the improved characteristics of such new formulations developed using a murine model will also improve efficacy in human CF patients.

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